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Impact of pre-inoculation of soybean seeds with *Bradyrhizobium* spp. applied 60 days before sowing

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Abstract

The pre-inoculation of soybean seeds optimizes sowing process, enhancing biological nitrogen fixation, seed quality, and overall crop yield. This study aimed to evaluate the efficacy of pre-inoculating soybean seeds with the commercial inoculant Biofix Premium (*Bradyrhizobium diazoefficiens* SEMIA 5080 and *Bradyrhizobium japonicum* SEMIA 5079, 5x10^9 CFU/mL), along with a cell protector and chemical treatment. Four soybean trials were performed in Paraná, São Paulo, Minas Gerais, and Goiás states during the 2021/2022 agricultural year, utilizing a randomized complete block design with four treatments and six replications. The treatments were: 1- Control (no inoculation); 2- Fertilization with 200 $kg.ha^{-1}$ of nitrogen; 3- Commercial standard seed inoculation (50 mL/50 kg of seeds) on the sowing day; 4- Seed inoculation with Biofix Premium (125 mL/100 kg of seeds), Protetor Ultra (0.5 mL/kg), Potenzial TS (0.2 mL/kg), and CoMo Platinum (100 mL/ha), applied 60 days before planting. Results showed that Biofix Premium applied 60 days before sowing, formed nodules and nodule mass similar to the comercial inoculant applied at sowing day. This led to a similar nitrogen fixation, chlorophyll content, shoot dry mass accumulation, grain yield, and nitrogen content compared to the comercial inoculant applied at sowing day. Yield gains with Biofix Premium ranged from 5-8% compared to the control (without inoculation), with no significant differences from the commercial seed inoculation applied on the sowing day. The use of insecticides and fungicides together with Biofix Premium, Protector Ultra, and Potenzial TS is recommended in the pre-inoculation of soybean seeds up to 60 days before sowing, without compromising plant nodulation and crop yield.

Keywords: Pre-sowing; Rhizobia; *Glycine max*; symbiosis; Nitrogen fixation; Crop yield.

Abbreviations: CFU_colony-forming unit, BNF biological nitrogen fixation, N_nitrogen, NNMR_number of nodules on the main root, DMNMR_dry mass of nodules on the main root, FMS_fresh mass of shoots, DMS dry mass of shoots, GY_grain yield, WTG_weight of a thousand grains, ATP_adenosine triphosphate, NADPH_reduced nicotinamide adenine dinucleotide phosphate, FCI_Falker chlorophyll index, TGW_thousand grain weight, PNG_percentage of nitrogen in grains.

Introduction

Brazilian soybean (*Glycine max* (L.) Merrill) production showed a geometric annual growth rate of 6.5% between 2011/2012 and 2021/2022 agricultural years, which doubled the volume produced, jumping from 66.4 million to 124.1 million tons. For this, two elements presented great importance: Both area and yield. The growing area increased significantly in the period, from 25.0 to 40.9 million hectares, as well as yield, increasing from 2.651 $kg.ha^{-1}$ in the 2011/2012 harvest to 3.029 $Kg.ha^{-1}$ in the 2021/2022 harvest. Of this total, 84.3% of the national soybean area is occupied by the south, southeast and midwest regions (CONAB, 2022).

The increase in soybean yield and relevance is directly linked to scientific advances and the availability of technologies to the productive sector (Xianzhong et al., 2022). An example of this is presented by Hungria et al. (2001), as genetic breeding associated with the selection of strains of nitrogen-fixing bacteria (BNF). Nitrogen (N) is responsible for increasing grain yield and protein content of soybean seeds (Ciampitti et al., 2021), in addition to its fundamental role in plant metabolism, which demands a high amount of N for amino acid biosynthesis, chlorophyll, nucleic acids and nitrogenous bases content (Jin et al., 2015).

Nitrogen can be provided through nitrogen fertilizers; however, to meet the crop demand for this element, it is necessary to provide a superior amount because a percentage of this nutrient is lost in the environment. Despite the low efficiency of chemical N, nitrogen fertilizers have high energy cost for their production (Hungria et al., 2001, Hungria et al., 2007). Currently, BNF is the main source of N for soybean crops. Bacteria of the genus *Bradyrhizobium* infect the roots of the plant via root hairs, forming nodules and providing the entire N demand for soybean crop (Hungria et al., 2007; Hungria and Mendes, 2015; Hungria and Nogueira, 2019).

In the 2019–2020 crop season, it was estimated that soybean BNF in Brazil resulted in saving more than $15.2 billion dollars annually on nitrogen fertilizers, contributing to the establishment of a more sustainable agricultural system (Telles et al., 2023). According to Câmara (2014), BNF supplies 70% to 95% of the N nutritional needs, so that 5% to 30% must be supplied by the soil and, mainly, by organic matter. This situation does not impose the need to fertilize the crop with mineral N, but the adoption of growing technologies that take into account crop rotation, since soybean plants are also higly efficient in using N from the mineralization of organic matter. Therefore, this process is boosted in growing systems with large straw or cultural residues (Souza et al., 2018).

Brazil is reference in the use of inoculants containing *Bradyrhizobium* spp., as these inoculants may contain a combination of two or more strains (Barbosa et al., 2021). Four strains of *Bradyrhizobium* are registered for commercial use in Brazil, which were isolated in growing sites from south (*B. elkanii* SEMIA 587), southeast (*B. elkanii* SEMIA 5019), and midwest (*B. diazoefficiens* SEMIA 5080 and *B. japonicum* SEMIA 5079) (Boddey and Hungria, 1997).

Other technological alternatives are studied to provide yield increments for soybeans and potentiate biological nitrogen fixation, such as pre-inoculation (Wang et al., 2022). Inoculation is often described as an activity that reduces sowing efficiency, due to the time spent on its operation, since most inoculants are applied at sowing day, generating extra work that demands time and labor, which may reduce its adoption by farmers (Campo and Hungria, 2007). The use of pre-inoculation may be a suitable alternative to improve crop management (Zilli et al., 2010; Hungria et al., 2020).

The inoculation of seeds as a industrial treatment has been used for some decades in other countries, for several crops besides soybean (Deaker et al., 2004; Herridge, 2008; Brzezinski et al., 2017) and it has become a strategy that tends to be spread to soybean cultivation in Brazil as well (Araujo et al., 2017). However, the implementation of this practice is influenced by several factors. These factors include the ability of bacteria to remain active on the seed, the storage conditions of the seeds, and the impact of other products added to the soybean seeds during inoculation (Anghinoni et al., 2017). According to several research works, a very important limiting factor lies in the rapid death of *Bradyrhizobium* spp. by fungicides, insecticides and micronutrients used in seed treatment (Hungria et al., 2007; Campo et al., 2009; Zilli et al., 2009). The most critical situation occurs with fungicides, as high mortality rates of *Bradyrhizobium* spp. have been observed, reaching 62% after just two hours and 95% after 24 hours (Campo et al., 2009). Consequently, the compatibility of the pre-inoculation must be carefully specified regarding the maximum time allowed, as well as the presence or absence and composition of fungicide, insecticide and micronutrient products.

In this context, the development of new inoculants and technologies for pre-inoculation, as well as the increase in time between seed treatment and sowing, has been increasingly sought by industries, resulting in many research studies of which several show promising results (Zilli et al., 2010; Araujo et al., 2017). In recent years, due to the demand from farmers for pre-sowing inoculants, several works on this topic have been performed, and registered products are already on the market, showing the viability of this technology (Hungria et al., 2020).

The aim of this study is to assess the viability and agronomic effeciency of the Biofix Premium inoculant, containing *B. diazoefficiens* - SEMIA 5080 and *B. japonicum* - SEMIA 5079, on seed treatment (pre-inoculation) of soybean crops. The study aims to evaluate the impact of the product on various performance parameters of the crop under field conditions.

Results

Symbiotic efficiency

The results for the variables number of nodules on the main root (NNMR), dry mass of nodules on the main root (DMNMR), fresh and dry mass of shoots (FMS and DMS) and Falker chlorophyll index (FCI) for all evaluated environments are available in Table 1.

In the municipality of Palmeira-PR, the NNMR ranged from 6.61 to 9.61 among treatments, within the 60 days anticipated seed treatment with Biofix Premium (Treatment 4) presenting a higher number in relation to the Treatment 2. However, it did not statistically differ from treatment 1 and Treatment 3. Regarding the variable DMNMR, treatment 4 stood out in relation to the others, presenting greater mass of nodules. Treatment 3 and treatment 1 did not differ from each other, and were statistically superior to treatment 2. The FMS did not significantly differ among tratments, with an average of 17.04 grams observed for treatment 1, and 21.42 grams for treatment 4. However for DMS, it is noted in Table 1 that treatment 2 was statistically superior to the others, while treatment 1, treatment 3 and treatment 4 did not statistically differ. For the FCI, the lowest index was observed for treatment 1. The treatment 4 inoculated with Biofix Premium, and treatment 3 did not statistically differ from each other and from the treatment 2 (Table 1).

In Itapira-SP, the average NNMR ranged from 14.33 to 46.72 among treatments, with the inoculated treatments (treatments 3 and 4) presenting higher values, differing from treatment 1 and treatment 2. The same statistical pattern was observed for the DMNMR, where the inoculated treatments presented an average mass of 153.1 and 164.72 milligrams, while treatment 2 presented mass of 44.9 grams. For both FMS and DMS, the inoculated treatments (treatment 3 and 4) were statistically equal to the nitrogen treatment (treatment 2), and presented higher values than treatment 1. The FCI ranged from 439.9 to 489.8, where treatment 4, treatment 3 and treatment 2 did not statically differ among themselves, while treatment 2 and treatment 3 differed from the treatment without inoculation (treatment 1).

In Araguari-MG, the inoculated soybean (treatments 3 and 4) showed higher production of nodules, with average above 80 NNMR, with treatments with pre-inoculation and inoculation on the sowing day statically not differing, standing out compared to the others. Lower NNMR was observed for treatment 1. Higher mass of nodules was also observed for treatments with inoculants (treatment 4 and treatment 3), which presented greater mass than treatment 2 and treatment 1. For the variables FMS and DMS, the treatment with early inoculation (treatment 4) did not statistically differ from treatment 3 and treatment 2 (commercial product, and nitrogen supplied treatments, respectively), being both statistically superior to treatment 1. Regarding FCI, treatment 4, treatment 3 and treatment 2 did not significantly differ from each other, but were superior to treatment 1 (Table 1).

In the municipality of Catalão-GO, the inoculated treatments (treatment 3 and 4) did not statistically differ from each other and showed nodulation superior to treatment 2 and treatment 1. The same behaviour was observed for the character DMNMR, where the inoculated treatments (treatments 3 and 4) presented greater mass, standing out in relation to the other treatments, with the nitrogen supplied treatment (treatment 2) and the control (treatment 1) presenting lower averages. The FMS presented no significantly differences for inoculated treatments (treatments 3 and 4). However, treatment 3 differed from treatment 1. Regarding DMS, both inoculated treatments (treatments 3 and 4) were statically equal and differed from the reatment 1. For FCI, the inoculated treatments did not statistically differ from each other, but differed from treatment 1, which presented lower chlorophyll index. The highest FCI was observed for Treatment 2, which was supplied with nitrogen fertilization (Table 1).

Production factors and nitrogen content in grains

In Palmeira-PR, the grain yield (GY) of the inoculated treatments (treatments 3 and 4) were statistically equal to each other and to treatment 2, but differed from treatment 1 (no inoculation), presenting yield increase of 5-7% compared to treatment 1. Regarding the character weight of a thousand grains (WTG), only the N200 treatment (treatment 2) differed from treatment 1. The N content in the grains (PNG) ranged from 5.35 to 5.46%, demonstrating that for inoculated treatments (treatments 3 and 4), the total N content was statistically equal to the nitrogen supplied treatment and superior to treatment 1 (Table 2).

Regarding the grain yield, in Itapira-SP, early inoculation of treatment 4 did not statistically differ from the treatment 3 inoculated on the sowing day and treatment 2, presenting increase of 8% compared to the Treatment 1. Regarding the WTG, there was no significant difference between inoculated treatments (treatments 3 and 4) and the nitrogen supplied treatment (treatment 2), but only treatment 3 differed from treatment 1. Regarding the PNG in the grains, the percentage ranged from 5.26 to 5.47%, which resulted in a higher N content per hectare in the inoculated (treatment 3 and 4) and nitrogen supplied (treatment 2) treatments, which differed from the treatment 1 (Table 2).

In municipality of Araguari-MG, the GY observed in the inoculated treatments was 6% higher than treatment 1, with no statistical differences between the pre-inoculation (treatment 4) and inoculation at sowing (treatment 3), which did not differ from the nitrogen supplied treatment (treatment 2) either. Regarding WTG, there were no statistical differences among treatments. The soybean grains presented percentages of nitrogen ranging from 5.43-5.52%, resulting in a total N production statistically higher in the treatments with inoculation or nitrogen fertilization than treatment 1 (control) (Table 2).

In Catalão-GO, all inoculated treatments (treatments 3 and 4) were statistically equal to treatment 2 for GY, and differed from treatment 1, with treatment 4 presenting increase of 6% in GY compared to treatment 1. For the WTG, the inoculated treatments did not statistically differ from treatment 2. However, only treatment 3 and treatment 2 differed from Treatment 1. The average value of nitrogen in the grains ranged from 5.45-5.55%, where the early inoculation treatment (treatment 4) statistically similar to treatment 2, with total N content of 234.4 kg.ha^{-1}, which was higher than treatment 1 (Table 2).

Discussion

In the four studied regions, nodules were formed in the plants. However, there was considerable variation among locations. In the municipality of Palmeira and Itapira, the population of rhizobia in the soil was 9.324x10^3/g, contributing to increased nodulation even in the treatment 1, which was not inoculated. Nish and Hungria (1996) explain that even in soils with a high population of *Bradyrhizobium* sp., the number and mass of nodules increased with inoculation. Conversely, in the regions of Araguari and Catalão, no rhizobia were identified in the soil, despite a history of soybean cultivation. As a result, the treatment 1 showed lower nodulation, while the inoculated treatments exhibited extremely efficient nodulation. Similar results were observed by Sandini et al. (2018), where the non-inoculated treatment presented lower number and mass of nodules.

The average number of nodules obtained in Palmeira did not exceed 10 nodules per plant, and the low nodulation could be attributed to the acidic soil (pH 4.8). Câmara (2014) explains that better FBN occurs in the pH range of 5.0 to 6.0 ($CaCl_2$). Nevertheless, the pre-inoculated treatment (treatment 4) showed nodulation statistically equal to treatment 3, which received inoculation at the sowing day, and superior to treatment 2, wich received nitrogen supplementation and no inoculation. Stecca et al. (2018) studied the effect of soil pH on pre-inoculated soybean seeds associated with an osmoprotector and observed that the use of seed protector increased the number and dry mass of nodules in more acidic pH soils, positively affecting grain yield. They observed an increase of 10.8% for seeds pre-inoculated 4 days before sowing and 8.3% for seeds pre-inoculated 7 days before sowing. The number of nodules in the pre-inoculated treatment (treatment 4) was equal to treatment 3 in all studied locations, demonstrating that the studied inoculant has the potential for early use up to 60 days before sowing.

The success of pre-inoculation is also attributed to the use of seed protectors, which ensures the survival of cells for a longer period in seeds treated with insecticides and/or fungicides (Silva et al., 2018). A recent study of pre-inoculation of soybean seeds treated with pyraclostrobin + thiophanate-methyl + fipronil with a bacterial protector showed that after 35 days of inoculation, it was possible to recover 1.13x10^3 CFU per seed (Araujo et al., 2017).

Another determinant factor for the quality of BNF is the mass of nodules formed, which was higher in the inoculated treatments (treatments 3 and 4) compared to the nitrogen supplied treatment (treatment 2) in all locations. The DMNMR in the Treatment 1 ranged from 26.72 to 105.22 mg.plant^{-1}, while in the Treatment 4, it ranged from 36.94 to 303.50 mg.plant^{-1}. According to Hungria et al. (2007), a well-nodulated soybean plant at the flowering stage should have between 15 to 30 nodules, or 100 to 200 mg of dry nodules per plant.

The application of nitrogen in the treatment 2 resulted in a reduction of NNMR and DMNMR in all evaluated locations. Similar results have been observed by other authors (Hungria et al., 2006; Zuffo et al., 2019). The stimulation for bacterial infection by *Bradyrhizobium* occurs under conditions of low nitrogen availability in the soil, inducing the necessary stress to produce isoflavones that activate the NOD gene factor (Subramanian et al., 2006).

Table 1. Number of nodules on the main root (NNMR), dry mass of nodules on the main root (DMNMR), fresh and dry mass of shoots (FMS and DMS), Falker chlorophyll index (FCI) in the four evaluated regions during the 2021/2022 crop season.

PALMEIRA-PR										
Treatments¹	NNMR²		DMNMR² mg		FMS² g		DMS² g		FCI²	
1	8.67	ab	26.72	b	17.04	a	3.75	b	437.97	b
2	6.61	b	15.72	c	23.22	a	5.77	a	503.23	a
3	8.50	ab	30.11	b	18.97	a	4.37	b	493.27	a
4	9.61	a	36.94	a	21.41	a	4.68	b	456.83	ab
C.V. (%)³	27.18		19.76		28.20		19.61		9.94	
ITAPIRA-SP										
Treatments¹	NNMR²		DMNMR² mg		FMS² g		DMS² g		FCI²	
1	34.11	b	104.94	b	28.58	b	6.65	b	439.90	b
2	14.33	c	44.89	c	33.92	a	7.41	a	489.77	a
3	46.72	a	164.72	a	33.41	a	7.30	a	486.77	a
4	42.83	a	153.06	a	32.06	a	7.25	a	479.53	ab
C.V. (%)³	21.13		22.49		7.63		3.62		9.23	
ARAGUARI-MG										
Treatments¹	NNMR²		DMNMR² mg		FMS² g		DMS² g		FCI²	
1	12.33	c	58.71	c	50.72	b	11.02	b	416.17	b
2	31.72	b	119.78	b	56.50	a	12.19	a	454.70	a
3	82.78	a	308.11	a	55.60	a	12.04	a	442.47	a
4	80.78	a	303.50	a	54.61	a	12.14	a	440.20	a
C.V. (%)³	22.08		11.07		5.66		6.76		3.56	
CATALÃO-GO										
Treatments¹	NNMR²		DMNMR² mg		FMS² g		DMS² g		FCI²	
1	7.83	c	105.22	c	52.29	c	11.62	c	258.00	c
2	27.67	b	135.82	b	59.16	a	13.89	a	342.50	a
3	46.33	a	203.78	a	55.84	b	12.41	b	301.10	b
4	49.22	a	203.31	a	55.08	bc	12.24	b	303.07	b
C.V. (%)³	24.56		7.42		5.04		4.55		5.28	

1. Treatments: 1- Control (no inoculation); 2- Fertilization with 200 kg.ha^{-1} of nitrogen, 50% at planting and 50% at flowering; 3- Commercial standard seed inoculation (50 mL/50 kg of seeds) on the sowing day; 4- Seed inoculation with Biofix Premium (125 mL/100 kg of seeds), Protetor Ultra (0,5 mL/kg), Potenzial TS (0,2 mL/kg), and CoMo Platinum (100 mL/ha), applied 60 days before planting. 2. Means followed by the same letter in the column do not significantly differ from each other according to Duncan's test at a 10% level of probability. 3. Coefficient of variation in percentage.

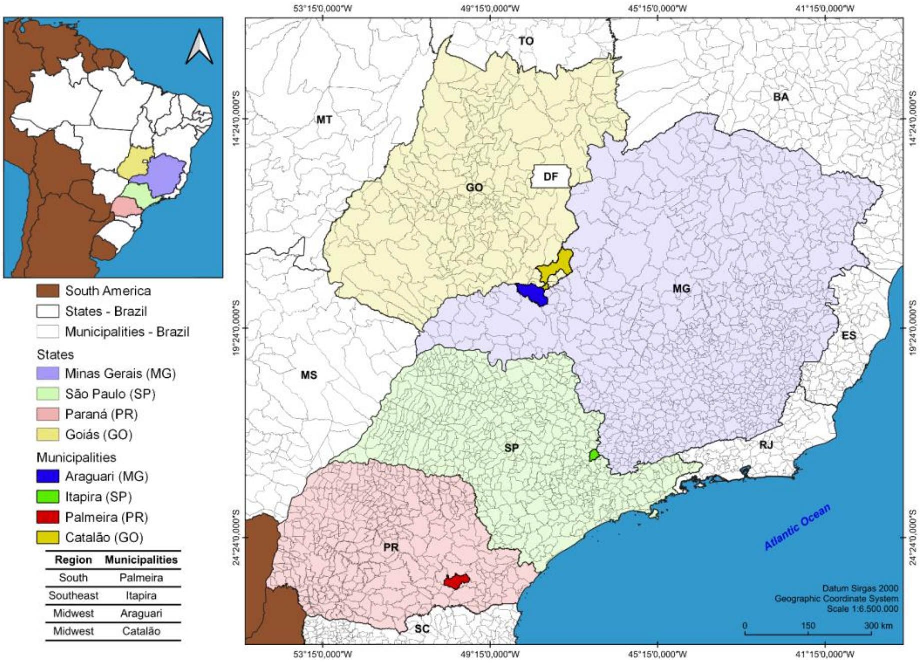

Figure 1. Distribution of testing sites.

Table 2. Factors of production: grain yield, relative production gain in percentage, weight of a thousand grains (WTG), percentage of nitrogen in the grains (PNG), and total nitrogen content (TNC) in the different regions evaluated during the 2021/2022 agricultural year.

PALMEIRA-PR								
Treatments[1]	Grain yield (kg/ha)		Relative gain (%)	WTG (g)		PNG (%)	TNC (kg/ha)	
1	2208.94	b	0	165.72	b	5.35	118.18	b
2	2312.29	a	5	172.60	a	5.46	126.25	a
3	2362.24	a	7	168.65	ab	5.45	128.74	a
4	2318.76	a	5	169.52	ab	5.43	125.91	a
C.V. (%)	4.28			2.97			4.29	
ITAPIRA-SP								
Treatments[1]	Grain yield (kg/ha)		Relative gain (%)	WTG (g)		PNG (%)	TNC (kg/ha)	
1	3509.54	c	0	129.23	b	5.26	184.60	c
2	3741.45	b	7	133.08	ab	5.47	204.66	b
3	3849.59	a	10	135.51	a	5.43	209.03	a
4	3796.01	ab	8	134.17	ab	5.46	207.26	ab
C.V. (%)	2.05			3.89			2.04	
ARAGUARI-MG								
Treatments[1]	Grain yield (kg/ha)		Relative gain (%)	WTG (g)		PNG (%)	TNC (kg/ha)	
1	3952.25	b	0	154.47	a	5.44	215.00	b
2	4112.06	ab	4	157.23	a	5.52	226.99	a
3	4191.42	a	6	157.41	a	5.48	229.69	a
4	4173.28	a	6	156.57	a	5.43	226.61	a
C.V. (%)	4.47			2.78			4.47	
CATALÃO-GO								
Treatments[1]	Grain yield (kg/ha)		Relative gain (%)	WTG (g)		PNG (%)	TNC (kg/ha)	
1	4040.97	b	0	171.55	b	5.47	221.04	c
2	4325.07	a	7	174.79	a	5.48	237.01	ab
3	4345.31	a	8	174.76	a	5.55	241.17	a
4	4300.53	a	6	173.70	ab	5.45	234.38	b
C.V. (%)	2.60			1.62			2.60	

1- Treatments: 1- Control (no inoculation); 2- Fertilization with 200 kg.ha^{-1} of nitrogen, 50% at planting and 50% at flowering; 3- Commercial standard seed inoculation (50 mL/50 kg of seeds) on the sowing day; 4- Seed inoculation with Biofix Premium (125 mL/100 kg of seeds), Protetor Ultra (0,5 mL/kg), Potenzial TS (0,2 mL/kg), and CoMo Platinum (100 mL/ha), applied 60 days before planting.

Table 3. Cultural treatments in each experimental region. 2021/2022 growing season.

Municipality	Pre-treatment date 60 days	Sowing date	Harvest date	Variety	Density (stes.ha^{-1})	Spacing (cm)	Predecessor culture	Rotation with soybean	Base fertilization	
									Source	Doses (kg.ha^{-1})
Palmeira - PR	02/12/2021	31/01/2022	23/05/2022	BMX Zeus	311.111	45	Wheat	Yes	Amino acids + 10-16-10	46.0 + 140
Itapira - SP	23/12/2021	21/02/2022	02/06/2022	BRS 245 RR	280.000	50	-	Yes	00-00-60 + 00-46-00	46.6 + 106
Araguari - MG	27/12/2021	24/02/2022	17/06/2022	N 7780 PRO	300.000	50	-	Yes	00-00-60 + 00-46-00	46.6 + 106
Catalão - GO	11/12/2021	09/02/2022	14/06/2022	Extrema IPRO	300.000	50	-	Yes	00-00-60 + 00-46-00	46.6 + 106

Table 4. Description of the four edaphoclimatic regions where the tests were conducted during the 2021/2022 growing season.

Region	Municipality	Latitude	Longitude
South	Palmeira - PR	25°25’49.40’’ S	50°03’15.31’’ O
Southeast	Itapira – SP	22°23’52.27’’ S	46°46’11.27’’ O
Midwest	Araguari - MG	18°31’51.53’’ S	48°02’59.85’’ O
Midwest	Catalão - GO	18°05’37.16’’ S	47°58’39.47’’ O

Table 5. Soil characteristics of each experimental region and initial rhizobia population in the soil. 2021/2022 growing season.

Municipality	Soil type	Texture Class	OM (g.dm^{-3})	pH (CaCl$_2$)	P (mg.dm^{-3})	Sand (%)	Silt (%)	Clay (%)	Rhizobium[2] (CFU.g soil^{-1})
Palmeira - PR	Alitic Haplic Cambisol	Silty Clay Loa	43.0	4.60	12.8	41.3	26.7	32.0	9.324.10^{3}
Itapira - SP	Dystrophic Red-Yellow Latosol	Clay	35.0	5.60	34.6	17.1	36.7	46.2	9.324.10^{3}
Araguari - MG	Dystrophic Red Latosol	Sandy Clay	36.0	5.30	3.30	51.3	11.5	37.2	0
Catalão - GO	Dystrophic Haplic Cambisol	Loamy Sand	14.0	5.90	3.00	84.7	7.6	7.7	0

1- Initial chemical and physical analysis of the soil conducted by the ABC Foundation; 2- Initial soil rhizobia population determined by the most probable number method, where CFU = colony-forming units.

Table 6. Climatic classification, average monthly precipitation and average monthly temperature of each experimental region. 2021/2022 growing season.

Municipality	Climatic classification[1]	JAN./22		FEB./22		MAR./22		APR./22		MAY./22		JUN./22	
		P (mm)	T[2] (º C)	P (mm)	T (º C)	P (mm)	T (º C)	P (mm)	T (º C)	P (mm)	T (º C)	P (mm)	T (º C)
Palmeira - PR	Cfb	254.5	22.3	39.0	22.6	149.5	21.6	98.3	19.9	99.5	15.8	-	-
Itapira - SP	Cfa	-	-	151.8	25.1	80.7	25.9	63.9	236	2.1	17.1	1.4	17.2
Araguari - MG	Aw	-	-	214.4	21.9	153.0	22.0	38.0	19.3	35.0	14.7	9.4	15.0
Catalão - GO	Aw	-	-	525.2	24.0	113.8	25.2	34.6	24.9	52.6	21.0	0.0	21.6

1. Climatic classification according to Köppen. 2. The monthly temperature values refer to the mean maximum and minimum for the month.

Table 7. Description of each treatment performed on soybean seeds. 2021/2022 growing season.

Evaluated treatments	Inoculant	Additive protectors[1]	Chemical treatment[2]	Pre-treatment days	Base fertilization (Kg.ha^{-1})
Treatment 1	-	-	Yes	-	-
Treatment 2	-	-	Yes	-	200
Treatment 3	Commercial standard inoculant	No	Yes	0	-
Treatment 4	Biofix Premium	Yes	Yes	60	-

1- Protectors, additives and fertilizers: Mixed mineral fertilizer Potenzial TS and Ultra protector. 2- The chemical treatments: Fungicide Maxin XL, and Insecticide Cruiser 350 FS.

Table 8. Description of biological products, additives, protectors and fertilizer, classes, active ingredients and doses used in soybean crop.

Commercial product	Class	Active ingredient	Dose
Commercial standard inoculant	Inoculant (commercial standard)	*B. japonicum* (Semia 5079 e 5080) 5,0.10^{12} CFU.mL^{-1}	1.0 mL.kg^{-1}
Biofix Premium	Inoculant (evaluated)	*B. diazoefficiens* (Semia 5080) e *B. japonicum* (Semia 5079) 5.10^{9} CFU.mL^{-1}	2.5 mL.kg^{-1}
Potenzial TS	Mixed mineral fertilizer	Mo 3,8%	0.2 mL.kg^{-1}
Protetor Ultra	Polyvinyl adhesive	-	0.5 mL.kg^{-1}
Maxim XL	Fungicide	Metalaxil-M 10g.L^{-1} + Fludioxonil 25g.L^{-1}	1.0 mL.kg^{-1}
Cruiser 350 FS	Insecticide	Tiametoxan 350g.L^{-1}	2.0 mL.kg^{-1}
CoMo Platinum[1]	Liquid fertilizer	-	100 mL.ha^{-1}

1- Foliar fertilizer applied at V3/V4 stage.

The chlorophyll content in the pre-inoculated treatment (treatment 4) did not statistically differ from treatment 3 in all evaluated regions, being superior to treatment 1 in the regions of Araguari and Catalão. Nitrogen plays a crucial role in several plant reactions and is a component of chlorophyll, enzymes, and proteins. Chlorophylls are involved in converting light energy into chemical energy in the form of ATP (adenosine triphosphate) and NADPH (reduced nicotinamide adenine dinucleotide phosphate) (Blankenship, 2009). Due to the high correlation between nitrogen content in plants and chlorophyll content (Argenta et al., 2001), this evaluation is valid to test the efficiency of diazotrophic bacteria (Meena et al., 2012). According to Vollmann et al. (2011), an increase in the number of nodules in plant is correlated with an increase in chlorophyll content in the leaves.

Regarding DMS, it was observed that in the regions of Itapira, Araguari, and Catalão, the inoculated treatments (treatment 3 and 4) differed from control (treatment 1), showing higher dry mass accumulation. In Itapira and Araguari, the inoculated treatments did not differ from the treatment 2.

Lower grain yield was obtained for treatment 1 in all evaluated regions, with treatment 4 presenting yield gains up to 8% in relation to treatment 1. Also, treatment 4 did not statistically differ from treatment 3 and treatment 2 in all locations. The average grain yield for treatment 4 ranged from 2318.76 to 4,300.53 kg ha^{-1}, while in the treatment 1, it ranged from 2208.94 to 4040.97 kg ha^{-1}.

For the weight of a thousand grains, treatment 4 did not statistically differ from the others in all locations, while treatment 3 showed higher values in the Itapira-SP region.

The accumulation of nitrogen in the grains was significantly higher in both inoculated (treatment 3 and 4) and nitrogen supplied (treatment 2) treatments, compared to control (treatment 1). In Palmeira, Itapira, and Araguari, there were no statistical differences between treatment 2 and treatment 4, all of which differed from treatment 1. In general, the higher number and mass of nodules in the inoculated treatments promoted greater nitrogen fixation, and, consequently, a higher amount of chlorophyll. These conditions led to an increase in the plant's photosynthetic rate, resulting in better shoot dry matter accumulation, grain yield, and nitrogen content in the grains.

The results obtained in the four locations corroborate with other studies previously carried out by Silva et al. (2018) studying the pre-inoculation of seeds treated with fungicides and insecticides 10 days before sowing in areas with and without history of soybean cultivation. The authors observed that pre-inoculation promoted nodulation, plant development and grain yield similar to the inoculant pattern in both places studied, also emphasizing the importance of protectors that enable the anticipation of inoculation. Machineski et al. (2018) verified that the pre-inoculation of soybean seeds using cell protector was efficient for keeping the bacterial inoculant viable in seeds for up to 60 days, and did not affect soybean productivity. Another similar study, carried out by Sandini et al. (2018), showed no impairment of nodulation and productivity in seeds treated within 71 days before sowing, in places with clayey soil and high organic matter content.

Hungria et al. (2020) reported that a liquid inoculant formulation applied 15 days before sowing provids an efficient BNF, ensuring greater time flexibility for producers, thus high soybean yields and N storage in the grains. Machineski et al. (2022) evaluated the agronomic efficiency of an inoculant applied 20 to 35 days before soybean sowing in four different locations. They reported that the grain yield and exported nitrogen content in the grains are statically similar, and even higher than the control without inoculation.

Material and methods

Field test methodology and crop management

Four soybean trials were conducted in the states of Paraná, São Paulo, Minas Gerais, and Goiás during the 2021/2022 agricultural year (Table 3 and 4), arranged in a randomized complete block design with four treatments and six replications. The distribution of testing sites is found in Figure 1. The size of each experimental plot was 24.8 m^2 (5 lines x 11 meters) with useful area of 12.2 m^2 (3 lines x 9 meters). The edaphoclimatic characteristics of the testing sites are described in Tables 5 and 6.

The fertilization used in the soybean crop during the tests were carried out in order to meet the needs of the crop for each region (Table 3). Other cultural treatments, such as herbicide, fungicide and insecticide applications, were carried out according to the needs of the crop (Seixas et al., 2020).

Plant material and evaluated characters

Soybean cultivars recommended for each region were used (Table 3). For each treatment, 4 kg of soybean seeds were treated using an appropriate machine to simulate the industrial seed treatment, in which fungicides and insecticides were sequentially added to all treatments. For treatment 4, we was also used the Protector Ultra and the mixed mineral fertilizer Potenzial TS, according to the stipulated pre-treatment period of 60 days, whereas the sowing of all treatments in the field test occurred within the schedule for each region studied. The volume of spray solution did not exceed the recommended 300 mL/50 kg of seeds. After seed treatment and complete drying, the seeds were placed in paper containers, stored in a dry place, away from light, with temperatures between 20 and 24 °C and air humidity below 70%.

Five plants with intact roots were collected from the central area of each plot, immediately before flowering (30 to 40 days after emergence), being evaluated the number of nodules on the main root per plant (NNMR), the dry mass of nodules in the main root (DMNMR) in milligrams per plant (mg plant^{-1}), the Falker chlorophyll Index (FCI) for determining the nitrogen (N) content in the shots, which was determined using a ClorofiLog Falker Chlorophyll Meter, model CFL1030, in 10 leaves of each replicate.

The collected plants were weighed to determine fresh mass of shoots (FMS) and after weighing, they were dried in an oven at 65 ºC, until they reached constant weight for determination of the dry mass of shoots (DMS), with data expressed in grams per plant. Grain yield (GY) was expressed in Kg.ha^{-1}, and the weight of a thousand grains (WTG) in grams, with data corrected to 13% moisture. The N content in the grains was evaluated by the ABC Foundation laboratory, which used the method proposed by Dumas (AOAC, 2005).

The initial concentration of rhizobia in the soil was determined by the analytical method approved by the Normative Instruction Nº30/2010 of 12/11/2010 (BRAZIL, Ministry of Agriculture, Livestock, and Supply of Brazil). It consists of inoculation of serial dilutions, in specific test plants (soybean plants), grown in aseptic conditions to evaluate the formation of nodules (MPN Technique – Most Probable Number).

Treatments description and data analysis

The treatments were:

Treatment 1: Control (no inoculation, and no nitrogen application);

Treatment 2: Fertilization with 200 kg.ha^{-1} of nitrogen, 50% applied at planting and 50% applied at flowering;

Treatment 3: Commercial standard seed inoculation (50 mL/50 kg of seeds), formulated with the bacteria *Bradyrhizobium japonicum* strains SEMIA 5079 and 5080, at concentration of 5x10^{9} CFU mL^{-1}, following the technical recommendation on the label, applied on the sowing day;

Treatment 4: Seed inoculation with Biofix Premium (125 mL/100 kg of seeds), a liquid inoculant composed of the bacteria *Bradyrhizobium japonicum* – SEMIA 5079 and *Bradyrhizobium diazoefficiens* – SEMIA 5080, at a final concentration of 5x10^{9} CFU mL^{-1}, combined with the following products: Protetor Ultra (0.5 mL/kg), Potenzial TS (0.2 mL/kg), and CoMo Platinum (100 mL/ha), applied 60 days before planting.

A description of treatments is also presented in Table 7, and all products used are presented in Table 8. The data was submitted to analysis of variance by the F test, and in the case of significant results, the difference between means was compared by the Duncan test at a 10% probability level, through the "software" Sasm - Agri (Canteri et al., 2001).

Conclusion

The pre-inoculation of soybean seeds with Biofix Premium up to 60 days before sowing produces nodules and mass of nodules statistically equal to the commercial product compared, which contributes to nitrogen fixation, accumulation of shoot dry mass, grain yield and nitrogen content in the grains, and also brings more flexibility of time for application and longer viability on the seeds, withstanding atypical conditions. The yield gains observed in the treatments with Biofix Premium ranged from 5-8% in relation to the control, not differing from the commercial inoculant applied on the sowing day.

The use of insecticides and fungicides associated with Biofix Premium, Protector Ultra and Potenzial TS can be recommended in the pre-inoculation of soybean seeds up to 60 days before sowing without compromising plant nodulation and crop yield.

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